REMARKS

In the Action, claims 14, 15 and 23-25 are rejected, and claims 16-22 are allowed. In response, claims 15 and 16 are amended, and claims 23-25 are cancelled. This leaves claims 14-22, with claims 14, 15 and 16 being independent.

Claim 15 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Amendment to claim 15 corrects the obvious clerical error and amends claim 15 to include the subject matter of original claim 14. Since this is the only rejection of claim 15, this amendment is submitted to place claim 15 in condition for allowance.

Claim 16 is also amended to be in independent form and to include the subject matter of original claim 14. Accordingly, claim 16 and the claims depending therefrom are believed to be in condition for allowance.

In view of these amendments and the following comments, reconsideration and allowance are requested.

The Rejections

Claims 23-25 are rejected under 35 U.S.C. § 102(b) as being anticipated by U.S. Patent No. 5,965,066 to Koch et al. By this Amendment, claims 23-25 are cancelled to obviate this rejection.

Claim 14 is rejected under 35 U.S.C. § 103(a) as being obvious over the literature article by Hirata et al. The Action cites the Abstract of the Hirata et al. article which discloses a single compound. Appended hereto is a complete copy of the literature article for the Examiner's consideration. The Action contends that the only difference between the compound of Hirata et al. and the claimed invention is the position of the isopropyl group on the phenyl ring and that it would have been obvious to produce the isomers of claim 14 by rearranging the positions of the substituents.

Hirata et al. provides no motivation, teaching or incentive to produce the claimed compounds of claim 14 which are starting compounds for preparing the final compounds of the invention recited in claims 15 and 16. Hirata et al. is specifically directed to an inhibitor of the tetracycline efflux pump in a tetracycline resistant clinical isolate of *Staphylococcus aureus* 743. In particular, Hirata et al. is directed to compounds that will increase the intracellular tetracycline concentration in the bacterial cell. Thus, Hirata et al. is directed to compounds having a specific bioactivity that by its nature is unpredictable. The bioactivity of compounds is specific to the substituent and its location. As noted in the Abstract of the Hirata et al. article, only three of the seven compounds examined exhibited a significant increase in the cellular concentration of tetracycline and where only two of the compounds appear to be energy inhibitors.

Hirata et al. is concerned with an unpredictable art as demonstrated by the compounds disclosed in Figure 3 and the bioactivity of the compounds shown in Figure 4. The unpredictable nature of the compounds disclosed by Hirata et al. provide no motivation or incentive to one of ordinary skill in the art to produce the position isomers as suggested in the Action. Moreover, Hirata et al. clearly provides no suggestion of rearranging the substituents according to the claimed invention. The bioactivity of the compounds disclosed by Hirata et al. are not inherently present in the claimed compounds and cannot be expected to have the same properties so that it would not have been obvious to one of ordinary skill in the art to modify the compounds of Hirata et al. Since Hirata et al. is concerned with the specific bioactivity of the compounds, it would not have been obvious to rearrange the compounds as in claim 14. Accordingly, the compounds of claim 14 are not obvious over Hirata et al.

In view of the above comments, claim 14 is submitted to be allowable over the art of record. Claims 15-22, as amended, are also submitted to be in condition for allowance. Accordingly, reconsideration and allowance of the claims are requested.

Respectfully submitted,

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Screening of an Inhibitor of the Tetracycline Efflux Pump in a Tetracycline-Resistant Clinical-Isolate of *Staphylococcus aureus* 743

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Clinically-isolated methicillin-resistant *Staphylococcus aureus* (MRSA) strain 743 exhibited resistance to tetracycline as judged from the active efflux of the drug. The efflux of tetracycline was inhibited by an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and minocycline. Inhibitors of the efflux pump were examined in this strain to determine the cellular accumulation of tetracycline. Out of seven compounds examined, three caused a significant increase in the cellular concentration of tetracycline by inhibiting the efflux pump. Two of them seem to be energy inhibitors. Ro 07-3149 inhibited the efflux pump without affecting the energy state, and exhibited very low antibacterial activity but showed weak synergy with tetracycline.

Key words *S. aureus*; tetracycline; drug resistance; inhibitor; drug efflux

Methicillin-resistant *Staphylococcus aureus* (MRSA) frequently shows antibiotic resistance against a wide range of antibiotics including tetracycline.[1] The resistance of MRSA to tetracycline is based largely on two resistance determinants, tet(M)[2] and tet(K).[3] The resistance mechanisms of tet(M) and tet(K) are based on ribosomal protection[4] and active drug efflux,[5] respectively. If an efficient inhibitor of the tetracycline efflux pump is found, it should be very useful for the eradication of MRSA.

Tetracycline enters bacterial cells *via* simple diffusion through the lipid bilayer region of the cytoplasmic membrane as a protonated neutral form and then is accumulated as a deprotonated negatively-charged form or a monocationic chelation complex with a magnesium ion, because the pH of the cell interior is higher than that of the exterior in energized cells.[6] Tetracycline efflux pumps, such as Tet(K)[5] and Tet(B),[7] actively export a tetracycline-divalent cation chelation complex through antiport with a proton.[8] Therefore, the tetracycline accumulation level of tetracycline-sensitive cells is high and deenergization by an uncoupler reduces the intracellular tetracycline concentration, whereas that of resistant cells is low and an uncoupler increases the intracellular concentration until there is a concentration equilibrium across the cell membrane.[9] On the other hand, an efficient inhibitor of the tetracycline efflux pump, if one exists, is expected to greatly increase the tetracycline accumulation level of resistance cells to that of energized sensitive cells.

S. aureus strain 743 is a typical MRSA strain, which was clinically isolated and showed tetracycline resistance. In this study, we found that *S. aureus* 743 has an active tetracycline efflux pump and that minocycline efficiently inhibits it. We used this strain to examine efflux pump inhibitors.

MATERIALS AND METHODS

Strain *S. aureus* 743 was clinically isolated in Nutley, New Jersey, U.S.A. in 1983, and is resistant to tetracycline as well as to many other kinds of antibacterial agents, including methicillin, erythromycin, trimethoprim and sulfomethoxazole.[1]

Reagents Muller-Hinton broth was obtained from Difco. [^{3}H]Tetracycline was purchased from DuPont-New England Nuclear. Ro 07-3149 (Fig. 3) was synthesized by Roche Nutley (NJ, U.S.A.), and its purity was more than 95%, as determined by thin layer chromatography. Other compounds were obtained from our in-house compound library. Other materials were all of reagent grade.

Assaying of [^{3}H]Tetracycline Uptake by Intact Cells *S. aureus* 743 cells were precultured with shaking overnight in Muller-Hinton broth supplemented with 20 μg/ml of tetracycline. One percent of the overnight culture was inoculated into the same medium and then the cells were grown to exponential phase (approximately 1.7×10^8 CFU/ml). The cells were harvested and washed once with the storage buffer (100 mM potassium phosphate (pH 7.2) and 7.5 mM ammonium sulfate), and then resuspended and concentrated twenty-times in the same buffer containing 200 μg of chloramphenicol/ml. The cells were stored on ice until use.

For the uptake assay, the cells were diluted three times with the assay buffer (100 mM potassium phosphate (pH 6.6), 100 mM KCl, 2 mM $MgSO_4$ and 0.4% glucose). Uptake was initiated by adding 20 μl of 25 μM [^{3}H]tetracycline (75 Ci/mole) to a mixture of 60 μl of the cell suspension and 20 μl of the inhibitor solution. After incubation at 37 °C for 80 min or the indicated times, the cells were filtered out on a glass filter and then immediately washed with the assay buffer. The radioactivity on the filter was counted with a Beta-Plate Counting System (Pharmacia). The intracellular concentration of tetracycline was calculated, with a counting efficiency of 56%, a cell volume of 1.3 μl/10^9 cells being used.

Antimicrobial Activity Antimicrobial activity was measured by the broth dilution method with Muller Hinton broth. Approximately 5×10^6 cells were inoculated into 100 μl of the broth in the presence of a series of 2-fold dilutions of the indicated drugs. The minimum inhibitory concentration was

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determined after cells had grown for 18 h at 37 °C.

RESULT

Active Efflux of Tetracycline from *S. aureus* 743 Cells *S. aureus* 743 showed antibiotic resistance to a wide variety of chemotherapeutic agents including tetracycline.[1] The accumulation of tetracycline in *S. aureus* 743 cells was very low when intact cells were incubated with [^{3}H]tetracycline (Fig. 1). The addition of an uncoupler, carbonyl cyanide m-chlorophenylhydazone (CCCP), increased the accumulation level by a factor of about 2. Minocycline, which is a hydrophobic derivative of tetracycline, greatly increased the cellular accumulation of tetracycline by a factor of about 30 when 400 μM minocycline was added (Fig. 1). These observations indicated that the intracellular concentration of tetracycline in *S. aureus* 743 was lowered by an active efflux pump and that minocycline is an efficient inhibitor of the efflux pump. The accumulation level of tetracycline was dependent on the minocycline concentration (Fig. 2). The max-

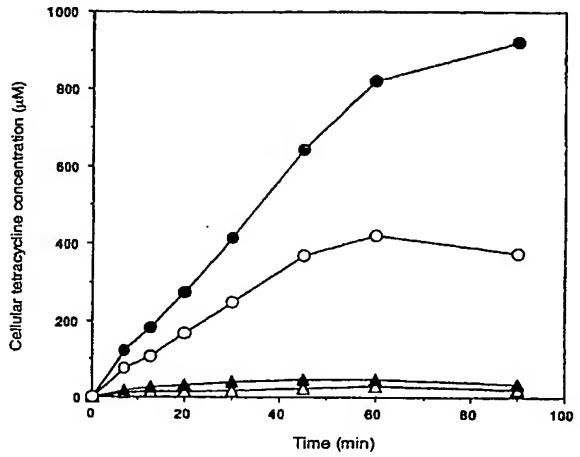


Fig. 1. Effects of Minocycline and CCCP on the Time Course of Tetracycline Accumulation in *S. aureus* 743 Cells

△, no drug added; ▲, 200 μM CCCP; ○, 200 μM minocycline; ●, 400 μM minocycline.

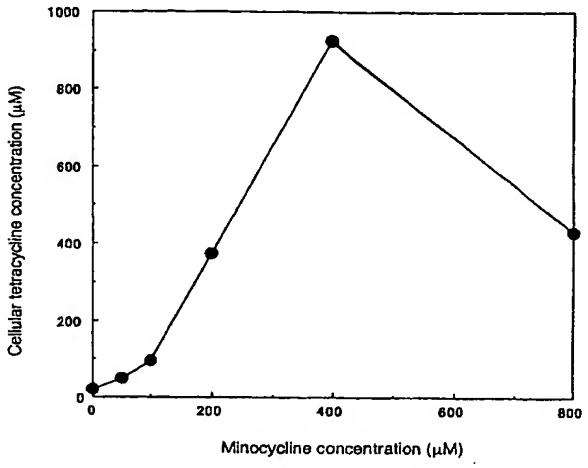
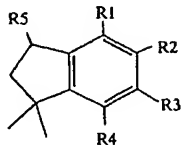


Fig. 2. Minocycline Concentration Dependence of the Intracellular Tetracycline Concentration in *S. aureus* 743 Cells

The tetracycline concentration was measured after 90 min incubation as described in Materials and Methods.



	R1	R2	R3	R4	R5
Ro 07-3149 (1)	CH_3	$-CH(OH)CH_2CH_3$	CH_3	CH_3	H
Compound (2)	$-CH(CH_3)_2$	H	OCH_3	CH_3	H
Compound (3)	CH_3	OH	H	CH_3	$-CH(CH_3)_2$
Compound (4)	$-CH(CH_3)_2$	H	OH	CH_3	H
Compound (5)	CH_3	H	OCH_3	CH_3	H
Compound (6)	CH_3	CH_3	OCH_3	H	H
Compound (7)	CH_3	H	OH	$-CH(CH_3)_2$	H

Fig. 3. Chemical Structures of Ro 07-3149 and Their Derivatives

(1), Ro 07-3149: 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan; (2), 4-isopropyl-6-methoxy-1,1,7-trimethylindan; (3),5-hydroxy-3-isopropyl-1,1,4,7-tetramethylindan; (4), 6-hydroxy-4-isopropyl-1,1,7-trimethylindan; (5), 6-methoxy-1,1,4,7-tetramethylindan; (6), 6-methoxy-1,1,4,5,-tetramethylindan; (7),6-hydroxy-7-isopropyl-1,1,4-trimethylindan.

Table 1. Effect of Ro 07-3149 on the Antibacterial Activities of Tetracycline toward Methicillin-resistant *S. aureus* 743 and a Drug Sensitive Wild-type *S. aureus* ATCC25923

Strain	MIC (μg/ml)				
	Ro 07-3149	Tetracycline	Tetracycline +8 μg/ml Ro 07-3149	Chloramphenicol	Chloramphenicol +8 μg/ml Ro 07-3149
S. aureus ATCC25923 (wild)	32	0.25	0.25	8	8
S. aureus 743 (MRSA)	32	64	32	8	8

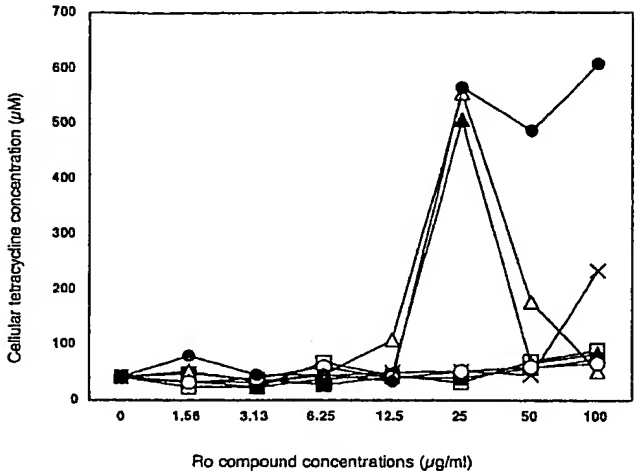


Fig. 4. Effects of Ro 07-3149 and Its Derivatives on the Intracellular Tetracycline Concentration in *S. aureus* 743 Cells

The tetracycline concentration was measured after 75 min incubation as described in Materials and Methods. ●, Ro 07-3149 (1); ○, compound (2); ▲, compound (3); △, compound (4); ■, compound (5); □, compound (6); ×, compound (7).

imum level was reached with 400 μM minocycline, and at higher minocycline concentrations the level inclined to decrease, probably due to minocycline-induced de-energization.

Effects of Various Compounds on the Accumulation Level of Tetracycline in *S. aureus* 743 Cells The effects of a novel compound, 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan, Ro 07-3149, and six derivatives of it on the intracellular accumulation level of tetracycline in *S. aureus* 743 were investigated (Fig. 4). Three of these compounds did not affect the accumulation of tetracycline. Compounds (3) and (4) increased the intracellular tetracycline concentration at 25 μg/ml, whereas at higher concentrations, the intracellular concentration of tetracycline decreased to the level without compounds (Fig. 4), probably due to de-energization. These results indicate that these two compounds are certainly efflux pump inhibitors but that they also act as uncouplers at high concentrations. Compound (7) moderately increased the intracellular tetracycline concentration at 100 μg/ml, indicating this compound is a weak inhibitor. Only Ro 07-3149 greatly increased the intracellular tetracycline concentration at 25 μg/ml, and at higher concentrations did not affect the level of this drug (Fig. 4). Ro 07-3149 seems to be an efficient efflux pump inhibitor without uncoupling activity because (1) significant accumulation of tetracycline in cells was observed when Ro 07-3149 was added whereas only a low concentration of tetracycline in cells was observed in the presence of the uncoupler, CCCP and (2) the level of accumulation was constant over the range of concentrations tested.

Effect of Ro 07-3149 on the Antibacterial Activity of Tetracycline As shown in Table 1, the antibacterial activity of Ro 07-3149 is weak. The minimum inhibitory concentration (MIC) for *S. aureus* 743 cells is only 32 μg/ml. On the other hand, the MIC of tetracycline for these cells is 64 μg/ml (Table 1), which is much higher than that for tetracycline-sensitive *S. aureus* ATCC25923 cells (0.25 μg/ml). In the presence of 8 μg/ml (1/4 MIC) of Ro 07-3149, the MIC value of tetracycline decreased two-fold, whereas that of chloramphenicol was unchanged (Table 1).

DISCUSSION

We showed in this study, that *S. aureus* 743 has an active tetracycline efflux pump and that Ro 07-3149 is an efficient inhibitor of this pump. The presence of the tet(K) gene was detected by the PCR method in DNA isolated from *S. aureus* 743 cells (data not shown). The combined effect of Ro 07-3149 with the antibacterial activity of tetracycline was observed but the tetracycline resistance of this strain was not lost in the presence of Ro 07-3149. Therefore, the ribosomal protection mechanism may also contribute to the tetracycline resistance of the strain.

We investigated the mechanism of the Ro 07-3149 action on the Tet(K) efflux pump expressed in *Escherichia coli* in detail in our previous study.[10] It was confirmed to inhibit the Tet(K) pump in everted membrane vesicles of *E. coli* without affecting the energy state of the membrane.[10]

The screening method described here is based on the fact that efflux pump inhibitors greatly increase the intracellular drug concentration. The level is far higher than that reached on de-energization by an uncoupler. This is a simple and very specific method for the screening of efflux pump inhibitors without an uncoupling action. This is a very important factor because usual methods based on the synergetic effects of inhibitors with the antibiotic actions of drugs tend to reveal energy inhibitors, most of which are not suitable for chemotherapy due to their toxicity. The combination of synergy and drug accumulation methods is the most preferable for the screening of efflux pump inhibitors.

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